Isolation and Biological Activity of Thielocins: Novel Phospholipase A_2 Inhibitors Produced by *Thielavia terricola* RF-143

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Thielocins A2α, A2β, A3, B1, B2 and B3 were isolated as a novel family of phospholipase A_2 inhibitors from the fermentation broth of *Thielavia terricola* RF-143 together with thielavins and thielocins A1α and A1β. The most potent inhibitory activity ($IC_{50}=0.0033\,\mu M$) against rat group II phospholipase A_2 was shown by thielocin A1β. Against human group II phospholipase A_2, thielocin B3 ($IC_{50}=0.076\,\mu M$) was the most potent.

Phospholipase A_2 (PLA_2) is a lipolytic enzyme which hydrolyzes the *sn*-2 fatty acyl ester bond of phospholipids to produce free fatty acids and lysophospholipids[1]. It exists in both extracellular and intracellular forms[2]. To date, two different extracellular PLA_2s have been identified based on their primary structures[3]: a pancreatic type (group I)[4] and a nonpancreatic "synovial" type (group II)[5]. Mammalian group I PLA_2 (PLA_2-I) is an important enzyme present in abundance in the digestive secretion of the pancreas[6], while mammalian group II PLA_2 (PLA_2-II) is found in inflammatory regions, such as casein-induced peritoneal fluid in rats[7], carrageenan-induced pleural exudate in rats[8] and synovial fluid of patients with rheumatoid arthritis[9]. These findings strongly implicate mammalian PLA_2-II as promoting inflammatory processes. In fact, some studies have shown the proinflammatory activities of PLA_2-II[10~12]. However, the pharmacological characterization and an understanding of the possible pathological roles of extracellular PLA_2s requires the development of the potent and specific inhibitors[13].

By using rat PLA_2-II, we searched for a specific inhibitor of PLA_2-II from culture filtrates of microorganisms and found thielocin A1β from the fermentation broth of *T. terricola* RF-143[14]. Thielocin A1β inhibited various PLA_2s in a dose-dependent manner. Among them, rat PLA_2-II was most sensitive to thielocin A1β ($IC_{50}=0.0033\,\mu M$)[15]. During further screening studies against the fermentation broth of *T. terricola* RF-143, we isolated several minor components as novel PLA_2 inhibitors together with the thielavins[16]. Their fermentation, isolation, and physico-chemical and biological characterization are described in this paper.

Materials and Methods

Materials

L-3-Phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]linoleoyl (2.18 GBq/mmol) was purchased from Amersham Corp. L-α-Phosphatidylethanolamine (from egg yolk), *p*-bromophenacyl bromide (*p*-BPB), mepacrine were purchased from Sigma. Manoalide was purchased from Wako Pure Chemical (Japan). Rat PLA_2-II was purified from rat platelets[17]. All other reagents were of analytical grade or better.

Chemistry

Melting points were determined on a Yanagimoto microscope hot-stage apparatus and are uncorrected. Ultraviolet spectra were taken on a Hitachi 323 spectrometer, infrared (IR) spectra (KBr pellet) on a Jasco DS-403G spectrometer, mass spectra on a Hitachi M-90 mass spectrometer, and proton nuclear magnetic resonance spectra on a Varian XL-400 spectro-meter in $CDCl_3$ solution with TMS as an internal standard. TLC was performed on pre-coated Silica gel $60F_{254}$ plates (E. Merck).

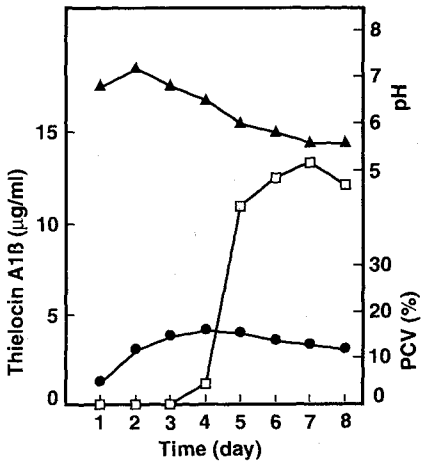
Fermentation

A slant culture of strain RF-143 was inoculated into a 2-liter Erlenmeyer flask containing one liter of a seed medium consisting of glucose 2.0%, polypepton 1.0%, beef extract 0.3%, yeast extract 0.2% and NaCl 0.1%. The flask was shaken at 28°C for three days on a rotary shaker at 180 rpm. One liter of the culture was transferred to a 30-liter jar fermentor containing 18 liters of the seed medium. The culture was incubated at 28°C for one day under agitation at 250 rpm and aeration of 10.8 liters per minute. For the production of thielocins 14 liters of the resulting culture fluid was transferred into 500-liter fermentor containing 350 liters of a medium consisting of soluble starch 2.0%, sucrose 2.0%, yeast extract 0.5%

in tap water and fermentation was carried out at 28°C for eight days under agitation at 320 rpm and aeration of 180 liters per minute. The back pressure of the fermentor was set at 0.35 kg/cm^2. All of the media preparations described above, pH of the medium was not adjusted. The time course of the production of thielocin A1β is shown in Fig. 1. The amount of thielocin A1β in the cultured broth was determined by analytical HPLC (Rt = 10 minutes; column: Cosmosil 5C_{18}-AR, 4.6 × 150 mm; solvent: 57% CH_3CN in 0.1% H_3PO_4; flow rate: 1.0 ml/minute; detection: 220 nm).

Fig. 1. Time course of thielocin A1β production.

The amount of thielocin A1β (□), pH of the medium (▲) and % of thielocin A1β in packed cell volume (PCV; ●) were measured for eight days.



Isolation and Purification

The isolation procedure for the thielocins is outlined in Fig. 2. Fractionation was guided by biological assay against rat PLA_2-II as described previously[18]. The mycelial cake was obtained by filtration of the harvest broth (356 liters) after adjustment to pH 2.5 with dil. HCl and extracted with acetone (108 liters). The acetone extract

Fig. 2. Isolation procedure for thielocins and thielavins.

Harvest broth (356 liters) of *Thielavia terricola* RF-143

↓ adjusted to pH 2.5 with dil HCl, filtered

Mycelium cake

↓ extracted with acetone (108 liters)

Extract (pH 3.8)

↓ concentrated *in vacuo*

Aqueous layer (30 liters)

↓ adjusted to pH 2.5 with dil HCl, extracted with AcOEt (25 liters)

AcOEt layer

↓ concentrated *in vacuo*, triturated with petroleum ether

Crude extract (448 g)

↓ dissolved in acetone (3.6 liters), addition of 200 mM phosphate buffer (pH 7.5, 8.4 liters)

CHP-20P column (20 liters)

1. acetone: 3% NaCl-20 mM phosphate buffer (pH 7.5), 3:7 (v/v) (10 liters)
2. acetone: 3% NaCl-20 mM phosphate buffer (pH 7.5), 3:7 (v/v) (30 liters)

⇩ linear gradient

acetone: 20 mM phosphate buffer (pH 7.5), 7:3 (v/v) (30 liters)

A (22.0 g)	B (24.6 g)	C (108 g)	D (17.6 g)
↓ Sephadex LH-20, prep. HPLC			
Thielocins A1, A2	Thielocins A3, A4, Thielocins B1, B2, B3	Thielavins A, B, C, D, E (68.3 g)	

Thielocins	A1	A2	A3	A4	B1	B2	B3
Amounts (mg)	1,050	400	30	10	20	20	30

was then concentrated *in vacuo* at pH 3.8. The resulting aqueous solution was extracted with ethyl acetate (25 liters) at pH 2.5. The AcOEt layer was concentrated *in vacuo* and the residue was triturated with petroleum ether to give a crude extract (448 g) which was then dissolved in a solvent mixture consisting of acetone (3.6 liters) and 20 mM phosphate buffer (PB; 20 mM Na_2HPO_4 was adjusted to pH 7.5 by 2 N HCl) (8.4 liters). The resulting solution was applied to a CHP-20P column (20 liters) which was packed and equilibrated with the same solvent mixture used to dissolve the crude extract. The column was eluted first with an eluent consisting of three volumes of acetone and seven volumes of 20 mM PB (pH 7.5) containing 3% NaCl (solvent A, 10 liters) and then eluted by linear gradient from solvent A (30 liters) to a solvent consisting of seven volumes of acetone and three volumes of 20 mM PB (pH 7.5) containing 3% NaCl (30 liters). The active fractions were divided into four fractions which were separately concentrated to give the partially purified fractions A, B, C and D. Each of the active fractions was further purified with a Sephadex LH-20 column developed with MeOH and then by preparative HPLC (column: YMC AP-324 s15/30 300A ODS, 50 × 500 mm, solvent; 70% CH_3CN in 0.1% H_3PO_4 for thielocins A1, B1, B2, 80% CH_3CN in 0.1% H_3PO_4 for A2 and B3). Each of the active fractions was concentrated and extracted with AcOEt to give pure thielocins A1 (1,050 mg) and A2 (400 mg) from fraction A, thielocins A3 (30 mg), A4 (10 mg), B1 (20 mg), B2 (20 mg) and B3 (30 mg) from fraction B, and thielavins A through E (68.3 g) from fractions C and D. Thielocin A1 fraction consisted of two isomers which were separated into thielocin A1α (220 mg) and A1β (830 mg) by preparative HPLC. Thielocins A2α and A2β were separated by preparative HPLC (column: YMC AP-324 s15/30 300A ODS, 50 × 500 mm; solvent: 68% CH_3CN in 0.1% H_3PO_4) to give pure A2α (164 mg) and A2β (236 mg). Thielavins A, B, C, D and E were separated by silica gel chromatography (Silica gel 60; Merck) eluted with AcOEt - MeOH (0→50%) and then with preparative HPLC using Nucleosil $7C_{18}$ (20 × 200) developed in a linear gradient from 55% CH_3CN in 0.1% H_3PO_4 to 70% CH_3CN in 0.1% H_3PO_4. Each of the active fractions was concentrated *in vacuo* and extracted with AcOEt to give thielavins A (0.710 g), B (50.35 g), C (12.01 g), D (0.81 g) and E (4.33 g).

Assay of Phospholipase A_2

PLA_2 activity was measured by a method described previously[15]. The substrate was prepared by diluting 1-palmitoyl-2-[1-^{14}C]linoleoyl phosphatidylethanolamine with L-α-phosphatidylethanolamine to the specific activity of 2,000 dpm/nmol. The reaction was started by addition of the enzyme. The amount of PLA_2s was adjusted to optimize linear kinetics for quantitation, *i.e.*, hydrolysis of the substrate was less than 20% in all experiments. Thielocins and other compounds were added to the assay tubes as a DMSO solution (2% of the final volume), using a DMSO-enzyme control. Control experiments showed that DMSO at this concentration had no effect on enzymatic activities. IC_{50} values were determined graphically from plots of percent inhibition, that were obtained from three independent experiments, each performed in duplicate, versus log concentration of test compounds.

Results

Taxonomy

The taxonomic properties of the strain RF-143 which produced the novel PLA_2 inhibitors can be summarized as follows. The vegetative hypha of strain RF-143 is macroscopically white in color on corn meal agar medium. The ascocarp which forms on the surface of the agar medium is spherical in shape and brownish black in color. It is 100 to 300 μm in diameter, and the textural

Table 1. Physico-chemical properties of thielocins A1α, A1β, A2α, A2β and A3.

	A1α	A1β	A2α	A2β	A3
Appearance	Colorless powder	Colorless powder	Colorless powder	Colorless powder	Colorless powder
MP	203~206°C	203~206°C	203~206°C	204~206°C	202~205°C
MW (MH^+; HR-MS)					
Found:	997.3861	997.3856	1175.4479	1175.4488	1175.4488
Calcd:	997.3854	997.3854	1175.4483	1175.4483	1175.4470
Molecular formula	$C_{54}H_{60}O_{18}$	$C_{54}H_{60}O_{18}$	$C_{64}H_{70}O_{21}$	$C_{64}H_{70}O_{21}$	$C_{64}H_{70}O_{21}$
UV (nm (ε))					
(MeOH and dil HCl - MeOH)			274.6 (25,100), 316.7 (5,380)	274.5 (22,900, sh), 321 (4,520)	274.5 (22,512), 318.6 (5,232)
(dil NaOH - MeOH)			260 (sh), 323.3 (52,800)	260 (sh), 323 (51,300)	255 (sh), 323 (29,825)
IR (KBr, cm^{-1})	3490, 1740, 1675 1577, 1155, 1095	3440, 1744, 1675, 1575, 1150, 1095	3440, 1740, 1605, 1574, 1150, 1090	3420, 1745, 1605, 1572, 1147, 1090	3440, 1743, 1657, 1608, 1576, 1097
HPLC (Rt)	7.0[a]	4.4[a]	9.7[a]	6.6[a]	16.9[b]

[a] Nucleosil $5C_{18}$, CH_3CN: 0.1% H_3PO_4 (69:31), detection; 230 nm (UV).
[b] Nucleosil $5C_{18}$, 60% aq CH_3CN containing 0.1% H_3PO_4.

Table 2. Physico-chemical properties of thielocins B1, B2 and B3.

	B1	B2	B3
Appearance	Colorless powder	Colorless powder	Colorless powder
MP	175~182°C	180~186°C	194~197°C
MW (MH^+; HR-MS)			
Found:	967.3754	967.3751	1131.4226
Calcd:	967.3749	967.3749	1131.4222
Molecular formula	$C_{53}H_{58}O_{17}$	$C_{53}H_{58}O_{17}$	$C_{62}H_{66}O_{20}$
UV (nm (ε))			
(MeOH and dil HCl-MeOH)	275 (24,150), 323 (5,120)	245 (24,830, sh), 325 (3,579)	276 (39,760), 306 (11,690, sh)
(dil NaOH-MeOH)	248 (21,250, sh), 331 (32,650)		
IR (KBr, cm^{-1})	3424, 1742, 1659, 1610, 1152, 1096	3440, 1775, 1734, 1665, 1610, 1150	3410, 1740, 1650, 1610, 1143, 1094
TLC (KGF plate)			
(Chf: MeOH, 2:1)	0.08	0.06	0.06
(Chf: EtOH: 10% AcOH, 4:7:2)	0.72	0.52	0.49
HPLC[a] (Rt)	5.0	3.0	7.2

[a] Cosmosil ODS-AR, 80% aq CH_3CN containing 0.1% H_3PO_4, detection: 230 nm (UV).

epidermoidea of the outer wall is brown. The ascus is $30\sim35\times15\sim17\,\mu m$ in size and is pyriform. The ascus which dissolves when matured, contains eight ascospores. The ascospores are broadly fusiform, olive to brownish gray in color, and $12\sim18\times6\sim8$ mm in size, with a germ pore at one end. The imperfect stage is absent. Based on these taxonomic properties, strain RF-143 was identified as *T. terricola*[19,20]. It has been deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession No. FERM BP-2196.

Physico-chemical Properties

The physico-chemical properties of thielocins are summarized in Tables 1, 2 and 3, and the ^1H NMR spectra are presented in Fig. 3. The structures of the thielocins were determined on the basis of the results of chemical degradation and spectroscopic characteristics as will be described in a separate paper. Thielavins A, B and C were identified according to the reported spectroscopic properies while thielavins D and E were found to be new compounds with closely related structures[16].

Table 3. Physico-chemical properties of thielavins D and E.

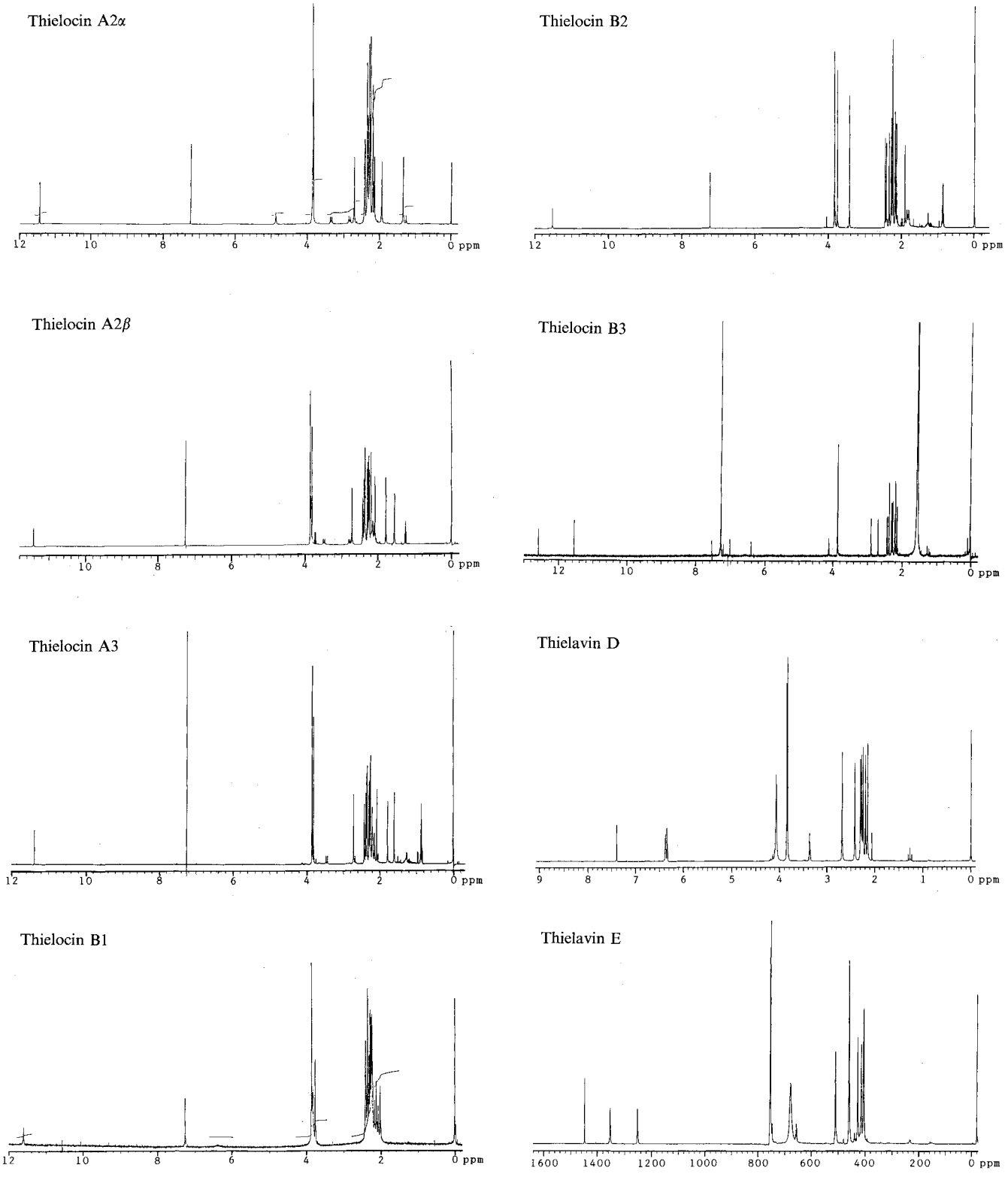
	D	E
Appearance	Colorless powder	Colorless powder
Molecular formula	$C_{30}H_{32}O_{10}$	$C_{30}H_{32}O_{10}$
MW (MH^+; SI-MS)	553	553
MP	235~238°C	217~220°C
UV (nm (ε))		
(MeOH)	269.5 (23,110), 303.3 (7,590)	275.9 (26,236), 305.0 (5,935)
(dil HCl-MeOH)	270.0 (23,050), 302.0 (46,417)	276.1 (24,679), 314.5 (43.205)
Rf value[a]	0.21	0.21
HPLC (Rt)[b]	7.90	10.2
Solubility		
Soluble	MeOH, EtOH, DMSO, Acetone, Pyridine, AcOEt, $CHCl_3$	
Insoluble	Petroleum ether, H_2O	
Slightly soluble	Benzene	

[a] Silica gel (Merk), AcOEt: MeOH, 2:1.
[b] Nucleosil $5C_{18}$ (4.6×150 mm), 58% aq CH_3CN containing 0.1% H_3PO_4; flow rate 1.8 ml/minute, detected at 230 nm.

Inhibition of Rat and Human Group II PLA_2 by Thielocins

We have already reported that thielocin A1β inhibited various extracellular PLA_2s in a dose-dependent manner. Among these, rat PLA_2-II was most sensitive to thielocin A1β[15]. As shown in Table 4, all other thielocins (thielocin A2α, A2β, A3, B1, B2 and B3) inhibited rat PLA_2-II more strongly than well-known PLA_2 inhibitors (mepacrine, *p*-BPB and manoalide). Nevertheless, thielocin A1β showed the most potent inhibitory activity against rat PLA_2-II ($IC_{50}=0.0033\,\mu M$). We have reported also that thielocin A1β showed weaker inhibitory activity against human PLA_2-II than rat PLA_2-II. As shown in Table 4, all other thielocins (thielocin A2α, A2β, A3, B1, B2 and B3) showed stronger inhibitory activity against human PLA_2-II than thielocin A1β and other PLA_2 inhibitors (mepacrine, *p*-BPB and manoalide). The strongest inhibition of human PLA_2-II among the thielocins was observed with thielocin B3 ($IC_{50}=0.076\,\mu M$).

Fig. 3. ^{1}H NMR spectra of thielocins and thielavins.



Discussion

Our data demonstrated that all the thielocins studied showed strong inhibitory activity against rat PLA_2-II (Table 4), the most potent being thielocin A1β (IC_{50} 0.0033 μM). As already reported, its inhibition was independent of both Ca^{2+} and substrate concentration and was also not affected by the substrate form (*Escherichia coli* membranes, phospholipids presented as surfactant mixed micelles or sonicated liposomes) and species of phospholipid used[15]. In addition, the inhibition of rat PLA_2-II by thielocin A1β was noncompetitive ($Ki=0.0068\,\mu$M) and reversible[15]. With regard to human PLA_2-II, thielocin A1β showed rather weak inhibitory potency with an IC_{50} value of 6.2 μM. Interestingly, thielocin B3 showed the most potent in-

Table 4. Inhibition of rat and human group II PLA_2 by thielocins.

Compounds	IC_{50} (μM)	
	Rat	Human[a]
Thielocin		
A1β	0.0033	6.2
A2α	0.051	0.31
A2β	0.038	0.24
A3	0.032	0.39
B1	0.0078	0.17
B2	0.070	2.7
B3	0.012	0.076
Thielavin		
A	43	29
B	1.3	2.4
C	0.46	2.1
D	1.1	6.2
E	4.5	9.3
Mepacrine	320	76
p-BPB[b]	6.7	34
Manoalide[b]	2.0	1.5

[a] Recombinant human group II PLA_2 was used.
[b] Both *p*-BPB and manoalide were preincubated with enzyme for 20 minutes at 37°C (pH 7.4).

hibitory activity ($IC_{50}=0.076\,\mu$M) against human PLA_2-II among those studied (Table 4). Furthermore, the double reciprocal plot showed that thielocin B3 and thielocin A1β behaved kinetically as noncompetitive inhibitors for human PLA_2-II with *Ki* values of 0.098 μM and 12 μM, respectively. Thus, thielocin B3 showed 120 times higher affinity for human PLA_2-II than thielocin A1β[21].

Several lines of evidence have suggested that PLA_2-II plays an important role in the pathogenesis of inflammatory disease[13]. In addition, some inflammatory cytokines and lipopolysaccharides dramatically increase PLA_2-II secretion in several tissues of rat through enhancement of gene transcription[22, 23]. Thus, PLA_2-II seems to participate in the development and possibly in the propagation of inflammatory processes. If such is the case, one might anticipate that inhibition of PLA_2-II would attenuate the severity of inflammation. Recently, we have reported[8] that at 5 hours after coinjection of thielocin A1β with carrageenan in the pleural cavity of rat, both the number of leukocytes and the amount of protein in the exudate had significantly decreased, and the exudate volume in the pleural cavity had decreased dose dependently ($ED_{50}=0.54$ mg/kg). Furthermore, PLA_2-II activity in the pleural exudate was decreased ($IC_{50}=0.060$ mg/kg) by coadministration of thielocin A1β. The decrease in PLA_2-II activity after various doses of thielocin A1β correlated well with the reduction in the exudate volume ($r=0.85$, $P<0.01$). These findings suggest that thielocin A1β possesses antiinflammatory activity, and its mechanism of action may contribute to the inhibitory activity of group II PLA_2. Of interest is the finding that thielocin B3, the most potent inhibitor against human PLA_2-II among the family of thielocins, also significantly decreased the exudate volume in the pleural cavity and PLA_2-II activity in the pleural exudate in a dose-dependent manner[21]. Recently, KAKUTANI *et al.*[24] reported that intravenous injection of the antibody, which inhibits the catalytic activity of PLA_2-II, significantly reduced both the pleural exudate volume and the leukocyte numbers in the pleural cavity. These observations indicate the important role of PLA_2-II in the pathogenesis of acute inflammation in the rat carrageenan-induced pleurisy model. However, this model might be far from the clinical situation. Hence, studies are in progress on the physiological role of extracellular PLA_2 in the progression of inflammatory processes in animal models that more exactly reflect the clinical situation using thielocins.

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